IgM-CAPTURE ELISA OF SERUM SAMPLES COLLECTED FROM FILIPINO DENGUE PATIENTS

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Abstract. Viral antigens for 4 dengue serotypes were produced in C6/36 *Aedes albopictus* cells. These were used as assay antigens for IgM-capture ELISA to detect IgM antibodies in sera of dengue patients from 3 hospitals in Metro Manila, Philippines. A total of 378 serum samples came from National Children's Hospital (NCH), San Lazaro Hospital (SLH), and St Luke's Medical Center (SLMC), from January to November 1995. Three hundred and four (304) out of 378 serum samples, or 80.42% showed positive IgM ELISA titer against at least one of the 4 assay antigens. Dengue type 4 (D4) antigen detected antibodies in 61.90% (234/378) of these serum samples, whereas type 1 (D1), type 3 (D3), and type 2 (D2) had detection rates of 60.05% (227/378), 50.79% (192/378) and 49.47% (187/378) respectively. Although the results show that both D1 and D4 are the most effective antigens in identifying dengue infections for this batch of samples, the use of a cocktail of antigens is still recommended. The results of this study are the basis for the IgM-capture ELISA protocol presently applied for the laboratory confirmation of dengue cases in the Philippines.

INTRODUCTION

Four different virus serotypes (D1, D2, D3 and D4) known to cause dengue fever and dengue hemorrhagic fever and distinguished on the basis of plaque reduction and neutralization tests, constitute a distinct antigenic complex (Monath and Heinz, 1996). They have been shown to share complex-specific or cross-reactive antigenic determinants, which serve to differentiate them from other flaviviruses (Henchal *et al*, 1982).

The current diagnosis of dengue infection in the Philippines relies mainly on clinical manifestation and classical serological tests. A dot-blot antibody assay that is commercially available is not sensitive enough to detect primary dengue cases (Fang *et al*, 1992). More reliable and efficient techniques are now available for the rapid diagnosis of infectious diseases. The development of IgM-capture ELISA (Burke, 1983) has provided a suitable alternative to the hemagglutination-inhibition test which has been used as the gold standard for confirmation of dengue diagnosis. Modifications of this technique (Bundo and Igarashi, 1985; Lam *et al*, 1987; Innis *et al*, 1989) have made significant advances in dengue serology.

One of the major drawbacks in using IgM-capture ELISA is the availability of dengue antigens needed for the assay. Assay antigens have been produced from infected suckling mouse brains by sucrose-acetone extraction (Clarke and Casals, 1958). Since the procedure requires animal handling and large quantities of organic solvents, it is laborious and time consuming (Igarashi *et al*, 1995). The establishment of the mosquito cell line *Aedes albopictus* C6/36 clone (Igarashi, 1978) provided a means of propagating the dengue virus in a target host cell (Soe Thein *et al*, 1979) rather than growing it *in vivo*. The production of high-titered dengue antigens *in vitro* does not require maintenance of mouse colonies and tedious extraction procedures. Moreover, this tissue culture-derived antigen has been found to be more sensitive than that derived from the suckling mouse brain (Mohamed *et al*, 1995a).

This study provides initial findings on the use of IgM-capture ELISA to detect antibodies from Filipino dengue patients.

MATERIALS AND METHODS

Test sera

Sera were collected from patients admitted in

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3 hospitals [National Children's Hospital (NCH), San Lazaro Hospital (SLH), and St Luke's Medical Center (SLMC)] all in Metro Manila, Philippines from January to November 1995. These patients were clinically diagnosed to have dengue virus infection. Sera were tested for the presence of antibodies against antigen from a specific dengue virus serotype (D1, D2, D3 or D4) by IgM-capture ELISA.

Production of assay antigen

Aedes albopictus clone C6/36 cells (Igarashi, 1978) were grown in 40 ml/Roux bottle of Eagle's medium in Earle's saline supplemented with 0.2 mM each of non-essential amino acids and 10% heat-inactivated fetal calf serum (FCS) at 28ºC. After 3 days, growth medium was removed from the cell culture and 1.5 ml of seed virus was inoculated. The following dengue virus strains were used in this study: D1, Hawaiian; D2, New Guinea B; D3, H83; and D4, No 17. Adsorption of the virus was done by spreading the inoculum over the cell sheet every 30 minutes for 2 hours at 28ºC. After adsorption, 40 ml of maintenance medium (growth medium containing 2% FCS) was added to the culture, which was then incubated at a specific temperature and time (Igarashi *et al*, 1994; Mohamed *et al*, 1995b; Kyaw-Sin-Thant *et al*, 1996), *ie* at 28ºC for 7 days for D1 or D4, at 37º C for 5 days for D2 and at 32ºC for 6 days for D3. After harvest of the infected culture fluid, viral antigen was detected by sandwich-ELISA following the micro-sandwich method of Voller and coworkers (1976). The antigen-ELISA titer of the harvested culture fluid was estimated by comparing its ELISA-OD with those of serially diluted standard positive specimens having predetermined endpoint titer (Igarashi *et al*, 1981; Morita *et al*, 1982). Harvested culture fluid of 9.6 units and above was used as standard assay antigen.

IgM-capture ELISA

Each step of the reaction in the IgM-capture ELISA was done at room temperature for one hour unless otherwise specified. Each well of 96-well flat bottom microtiter plates was coated with 100 µl of 11.75 µg/ml goat anti-human IgM (Organon, Teknika, NC, USA) in 0.05 M carbonate-bicarbonate buffer, pH 9.6 containing 0.01% NaN_3 for one hour at room temperature or at 4ºC overnight. Wells were then blocked with 100 µl of Blockace (Yukijirushi, Japan), followed by washing with PBS-Tween (0.05% Tween 20 and 0.01% NaN_3 in phosphate-buffered saline, pH 7.4) three times at three-minute intervals. One hundred µl of each test serum and negative control serum diluted 1:100 in PBS-Tween were pipetted into duplicate wells. The plates were washed as above. One hundred µl of infected culture fluid containing antigen from a specific virus serotype (D1, D2, D3 or D4) was added to each well. After washing as above, each well was reacted with 100 µl of horseradish peroxidase (HRPO)-conjugated antiflavivirus IgG in PBS-Tween. The reagent was prepared by conjugating the antiflavivirus IgG with HRPO (Sigma, type VI, USA), using Wilson and Nakane's method (1978). Wells were washed as above and the color reaction was done by adding 100 µl of 0.5 mg/ml *o*-phenylenediamine dihydrochloride (OPD) and 0.02% of H_2O_2 in 0.05 M citrate phosphate buffer, pH 5. After one hour of incubation in the dark, the reaction was stopped by adding 100 µl of 1 N sulphuric acid to each well. The OD_{450} on each well was measured by an ELISA reader with 620 nm as the reference wavelength. Test serum was considered positive when the average reading of the duplicate wells was at least twice higher than the average reading for the negative sera (P/N ratio ≥ 2).

Statistical analysis

The Cochran Q test was used to determine whether the use of different assay dengue antigens or any combination of 2 or 3 of them differ significantly in detecting dengue positive cases. The formula used was

$$Q=[k(k-1)\sum_{j=1}^{k}(Gj-\bar{G})^2] \div [k\sum_{i=1}^{N}L_i - \sum_{i=1}^{N}L_i^2]$$

where k = number of treatments
Gj = total number of successes in the jth column (jth category)
$\bar{G}$ = mean of Gj
L_i = total number of successes in the ith row (ith subject)
N = total number of observations/subjects

The SPSS software (Statistical Package for Social Sciences) was used to analyze the data.

RESULTS

Table 1 shows that 304 (80.42%) out of 378 serum samples were positive against at least one of the viral assay antigens (D1, D2, D3, or D4)

used. Table 2 indicates the cross-reactivity of the samples to the different viral assay antigens. One hundred four (34.21%) sera were found to be reactive to all assay antigens whereas 84 (27.63%) and 56 (18.42%) were reactive to 3 or 2 assay antigens, respectively. On the other hand, 60 (19.74%) reacted with only one serotype.

Table 3 gives the total number of samples reactive to at least one of the assay antigens. From this table, the assay antigen(s) that can maximally identify dengue positive patients can be noted. D4 can identify 234 cases out of 378 (61.90%), followed by D1 (227/378 or 60.05%), D3 (192/378 or 50.79%), and lastly by D2 (187/378 or 49.47%). Combining the number of positive cases for any 2 assay antigens, D1 and D4 can confirm 285 cases out of 378 (75.40%), followed by D1 and D3 (277/378 or 73.28%), D3 and D4 (265/378 or 70.11%), D1 and D2 (259/378 or 68.52%), D2 and D4 (254/378 or 67.20%) and lastly D2 and D3 (248/378 or 65.61%). For any 3 assay antigens, D1, D3 and D4 can detect 299 out of 378 cases (79.10%) followed by D1, D2 and D4 (291/378 or 76.98%), D1, D2 and D3 (289/378 or 76.46%) and lastly D2, D3 and D4 (277/378 or 73.28%). Statistical analysis (Cochran Q test; Siegel and Castellan Jr, 1988) in Table 4 shows that there is inequality in the ability of the assay antigens, either alone or in any combination of 2 or 3 different antigens, to identify dengue cases. Results (Tables 3 and 4) show that D2 and D3 do not differ in their reactivities and have a lower number of identified positive cases than D1 and D4. Similarly, D1 and D4 do not differ with each other with respect to their ability to confirm dengue infection. For a combination of 2 antigens, D1D4 and D1D3 can significantly identify more positive cases than the other remaining combinations. If 3 assay antigens are considered, there is no significant difference in the ability of the combinations of D1D2D3, D1D2D4 and D1D3D4 antigens in identifying positive cases. However, all these combinations are significantly more effective than that of the combination of D2D3D4. The combined ability of 4 antigens

Table 1
IgM-capture ELISA of serum samples using dengue serotype-specific antigens (D1, D2, D3, or D4).

Results of ELISA	Number	% of Total
Positive to at least one antigen	304	80.42
Negative to all antigens	74	19.58
Total number of samples	378	100.00

Table 2
Cross-reactivity of serum samples to the different viral antigens using IgM-capture ELISA.

Reactivity to different assay antigens (AA)	Assay antigens				No. of reactive samples for the different AA	Total no. of samples positive to a combination of AA
	D1	D2	D3	D4		
Reactive to 4AA	+	+	+	+	104	104 (34.21%)
Reactive to 3AA	+	+	+	-	7	84 (27.63%)
	+	+	-	+	37	
	+	-	+	+	21	
	-	+	+	+	19	
Reactive to 2AA	+	+	-	-	7	56 (18.42%)
	-	+	+	-	1	
	-	-	+	+	17	
	+	-	+	-	10	
	+	-	-	+	14	
	-	+	-	+	7	
Reactive to 1AA	+	-	-	-	27	60 (19.74%)
	-	+	-	-	5	
	-	-	+	-	13	
	-	-	-	+	15	
Total number of positive samples					304	304 (100%)

Table 3
Total number of serum samples reactive to at least one of the given assay antigens.

Assay antigens	Serum samples reactive to at least one assay antigen	% of positive samples out of the 378 samples tested
Single antigen		
D4	234	61.90
D1	227	60.05
D3	192	50.79
D2	187	49.47
Combination of 2 antigens		
D1D4	285	75.40
D1D3	277	73.28
D3D4	265	70.11
D1D2	259	68.52
D2D4	254	67.20
D2D3	248	65.61
Combination of 3 antigens		
D1D3D4	299	79.10
D1D2D4	291	76.98
D1D2D3	289	76.46
D2D3D4	277	73.28
Combination of all 4 antigens		
D1D2D3D4	304	80.42

Table 4
Results of the Cochran Q test of comparing proportions of positive results in detecting dengue cases by using one or a combination of assay antigens.

No. of assay antigen (s) compared	Test	Q-value	Significance
1 antigen	$Ho:P_{D1}=P_{D2}=P_{D3}=P_{D4}$	31.427	0.000[a]
	$Ho:P_{D1}=P_{D3}=P_{D4}$	17.460	0.000[a]
	$Ho:P_{D2}=P_{D3}$	0.137	0.712
	$Ho:P_{D1}=P_{D4}$	0.330	0.565
2 antigens	$Ho:P_{D1D2}=P_{D1D3}=P_{D1D4}=P_{D2D3}=P_{D2D4}=P_{D3D4}$	36.195	0.000[a]
	$Ho:P_{D1D4}=P_{D1D3}=P_{D3D4}$	8.686	0.013[a]
	$Ho:P_{D1D4}=P_{D1D3}$	1.361	0.243
	$Ho:P_{D1D2}=P_{D2D4}=P_{D2D3}=P_{D3D4}$	5.352	0.148
	$Ho:P_{D3D4}=P_{D1D2}=P_{D2D4}=P_{D2D3}=P_{D1D3}$	17.007	0.002[a]
3 antigens	$Ho:P_{D1D2D3}=P_{D1D2D4}=P_{D1D3D4}=P_{D2D3D4}$	16.533	0.001[a]
	$Ho:P_{D1D2D3}=P_{D2D3D4}=P_{D1D2D4}$	6.255	0.044[a]
	$Ho:P_{D1D2D3}=P_{D1D2D4}=P_{D1D3D4}$	5.091	0.078
3 antigens against 4	$Ho: P_{D1D3D4}=P_{D1D2D3D4}$	3.200	0.063

[a]Ho rejected at 5% level of significance.

(80.42% positive cases) is not significantly different from that of the combination of 3 antigens (D1, D3 and D4) that gave the highest number of positive cases (79.10%).

DISCUSSION

The present study applied the technique of ELISA to detect IgM antibodies from sera of

[a]Ho rejected at 5% level of significance

Filipino dengue patients. Four different assay antigens (D1, D2, D3 and D4) were produced from C6/36 *Aedes albopictus* cells infected with the 4 dengue serotypes respectively. Comparison of the reactivities of the patient sera against each of the four assay antigens showed that D1 and D4 antigens can identify more positive cases than either D2 or D3. To achieve maximum detection frequency, the use of a cocktail of all the antigens is necessary. From the results of this study, if 2 antigens are used in combination, a higher frequency of detection of positive cases compared with using only 1 antigen can be obtained. Similarly, using all 4 antigens will increase the detection to a maximum. However, statistical tests showed that with the 378 samples included in this study, the use of 3 dengue antigens (D1, D3 and D4) does not differ significantly from the use of 4 antigens. Currently, the ELISA protocol being used in the Philippines to identify dengue cases makes use of a cocktail of all 4 assay antigens. Further tests with a more extensive serum collection will be conducted to determine if a cocktail of three antigens will be as effective as using 4 antigens to confirm dengue positive cases.

Kyaw-Zin-Thant *et al* (1996) tested IgM-capture ELISA in detecting positive cases among 70 clinically diagnosed dengue patients admitted to a hospital in Union of Myanmar in 1994. They found that 62 out of 70 serum samples from these patients showed positive IgM ELISA titer against at least one of the 4 assay antigens. Single application of D2 antigen could detect 59 out of the 62 positive cases whereas D1, D3 and D4 could detect 48, 47, and 32 positive cases, respectively. They noted that the high-titered dengue antigens such as D2 (64 units) could detect a higher number of positive serum samples than the low-titered ones such as D4 (4 units). To find out the correlation between antigen ELISA units and the percentage of positive samples that could be detected, this same group of researchers (Khin-Mar-Aye *et al*, 1996) conducted an experiment on 33 paired serum samples (acute and convalescent sera) from patients previously identified by hemagglutination-inhibition test to be infected with dengue. They used D2 antigen for IgM ELISA and found that when the titer was reduced from 64 units to 32U, 16U, 8U and 4U, the percentage of positive samples was also reduced from 86 to 79, 62, 52, and 26 respectively. Thus, in detecting dengue positive cases through IgM-capture ELISA, it is important to initially establish the best antigen titer.

While IgM ELISA is able to provide results even with a single serum specimen (Igarashi, 1994), the method cannot identify the serotype of the infecting virus. Cross-reactivity is clearly observed in the use of antigen derived from culture fluid of C6/36 cells infected with a particular dengue serotype. Monoclonal antibodies are now available to detect specific serotypes. However, virus isolation followed by genome detection by PCR is still the most direct way of demonstrating the presence of the virus and identifying its serotype.

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